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## A. Background and Significance

Uncontrolled cell-extracellular matrix (ECM) interactions result in matrix turnover and tumor invasion. The cell-ECM interactions are coordinated series of events composed of modification of cell-cell binding and cell-ECM adhesion, proteolytic degradation of ECM and cell de-attachment, migration through ECM and entering into the circulation. The integrin cell surface adhesion bind to matrix proteins play important role in these processes. When unchecked, these interactions result in over-proliferation, circumvention of apoptosis and thus tumor growth and metastasis [1,2 and 27,28]. Matrix metalloproteases (MMPs) and membrane-type metalloproteases (MT-MMPs) also constitute one of the pivotal points of ECM turnover [3-6]. MMPs and MT-MMPs are intimately involved in the malignant phenotype as shown by numerous studies including those using the human breast carcinomas. This current study focuses on a newly cloned membrane protein, ADAMx, which contains both a metalloprotease domain and an integrin binding domain. The identification of this protease protein in the breast carcinoma cells suggests that ADAMx may play a role in breast tumor metastasis and invasion and the likely mechanism of action is through ADAMx - integrin interaction and the metalloprotease activities.

The ADAMs are a family of complex membrane proteins which mostly resembles the MT-MMPs (Figure 1). ADAMs are usually composed of a pro-protein domain (PP), a metalloprotease domain (MP), a disintegrin-like domain (DIS), a cysteine-rich domain (CR), a transmembrane domain (TM) and a cytoplasmic tail (CT). Some of the ADAMs also have EGF-like motifs (EGF) and fusion peptides (FP). To date, about 20 members of the ADAM family proteins are cloned from a variety of species and tissues [7-9].

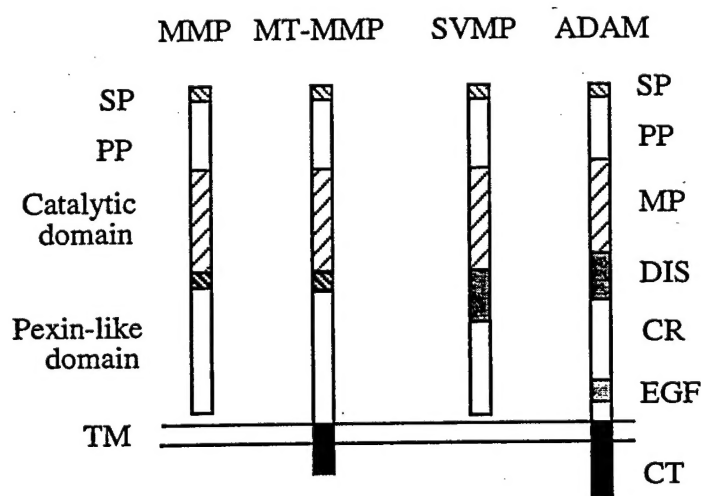


Figure 1. Domain arrangement of several types of metalloproteases

The ADAMs' PP and MP domains have the characteristics of MMPs and MT-MMPs, that is, most MP domains contain the consensus zinc-binding sequence HEXXH and many ADAMs also contain a potential "cysteine-switch" cysteine residue in the PP domain. Thus, like the MMPs and MT-MMPs, the protease activity in ADAMs may also be controlled by the "cysteine switch" mechanism [10,11].

The DIS domain of the ADAMs share striking structural homology to the snake venom peptide disintegrins, as demonstrated by their highly conserved locations of the cysteine residues. The snake venom disintegrins, which contain the RGD/KGD "integrin recognition sequence", are ligands for integrins [12,13]. Thus, the ADAMs are suggested to be integrin ligands. Unlike the peptide disintegrins, though, most ADAM disintegrins lack the RGD/KGD sequence in the presumed integrin-binding hairpin loop. In fact, the corresponding residues in the ADAM disintegrins are quite degenerate. One explanation is that the structure and the position of the loop, not the exact amino acid sequence, is key for integrin binding [14]. This is supported by the fact that mouse fertilin- $\beta$ , an ADAM protein, binds to integrin  $\alpha 6 \beta 1$  using the TDE sequence within the disintegrin domain [15].

What are the biological functions of the ADAMs? To date, most data about the ADAMs are generated from cell fusion studies. It is reported that ADAMs fertilin- $\alpha$  and  $\beta$  participate in sperm-egg fusion [7,15,16]. It is reported that during the sperm-egg fusion, the fertilin- $\beta$  binds to integrin  $\alpha 6 \beta 1$ , resulting in the exposure of the fusion peptide in the fertilin  $\alpha$ -subunit which, in turn, allows the fusion. ADAMs are also implicated in cell differentiation. For example, mouse meltrin- $\alpha$  was shown to play important role in myoblast differentiation and fusion, leading to myotube formation [7,17]. The latter is an important process in skeletal muscle generation. Another interesting function of the ADAMs is proteolysis of degradation and remodeling of surface proteins and ECM components, as the closely related snake venom metalloproteases (SVMP) do [7]. One study showed that TACE (tumor necrosis factor- $\alpha$  converting enzyme), an ADAM protein, can induce the release of TNF- $\alpha$  from tumor cells, presumably by cleaving membrane-bound TNF- $\alpha$  precursor [18]. Another ADAM, ADAM-11, is a candidate human cancer tumor suppressor, as its gene is disrupted in both the breast and ovarian cancers by somatic rearrangements [19]. In conclusion, the ADAM family proteins are likely involved in interactions with ECM components and with cell surface proteins, including the integrins. Therefore they most likely play functional role in tumorigenesis.

I have cloned a novel ADAM protein (ADAMx) from non-invasive human breast epithelial cell line MCF-7. ADAMx is not found in the MDA-MB-231 or MDA-MB-435 human breast carcinomas which have mesenchymal (fibroblast-like) morphology and are highly invasive [20,21]. This expression pattern is opposite to that of the MMPs and MT-MMPs, both of which are found to be expressed in the invasive cells, but not in MCF-7 cells [22]. The difference in the expression patterns leads to the hypothesis that ADAMx, as part of the ECM remodeling machinery, may act through a pathway different from that of the MMPs and MT-MMPs. It also suggests that ADAMx may be important for maintaining breast cell epithelial morphology and that the loss of ADAMx could link to tumor progression and metastasis.

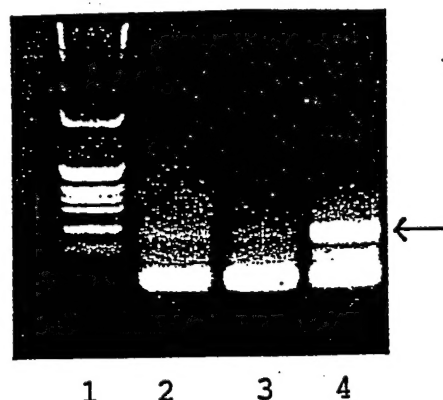
## B. Experimental Methods and Results

### B.1. Identification of ADAMx From Breast Cancer Cell Lines

To study the correlation between ADAM gene expression and tumor invasiveness, I have initiated experiments to identify ADAMs in several human breast cancer cell lines, including MDA-MB-435, MDA-MB-231 and MCF-7. The 435 cells represent advanced tumors because they are highly invasive, the 231 cells are moderately invasive and the MCF-7 cells are not invasive; Thus the latter cell line represents early stage tumors [20,21]

The initial cloning was performed by homology RT-PCR method using total RNA from the three cell lines, oligo dT<sub>20</sub> and a pair of degenerate primers derived from conserved sequences within ADAMs' disintegrin domains. The sequence of the primers are: sense (DIS-1): 5'-RSD-GAR-SAG-TGT-GAY-TGT-GG-3' and antisense (DIS-2): 5'-GCA-AWW-TTC-WGG-RAR-RTC-RCA-3'. Both sequences were derived from the respective conserved peptide sequence GEECD CG and CDLPE(L/H)C within the disintegrin domain of known ADAMs. The primers were synthesized by Integrated DNA Technologies Inc.. The reverse transcription reactions were carried out as follows: total RNA sample (1-5 µg) from the cells was incubated with oligo dT<sub>20</sub> (100-500 ng) and an aliquot of H<sub>2</sub>O at 65°C for 10 minutes, then at room temperature for 2 minutes and cooled down on ice. A final reaction volume of 20 µl containing this mixture, RNase Inhibitor, dNTPs, sodium pyrophosphate, reverse transcription buffer (10x) and 0.5 unit of AMV reverse transcriptase (Promega) was put in a thermal cycler and the reverse transcription (RT) reaction was carried out at 45°C for one hour. The reaction tube was heated at 95°C for 2 minutes and quickly chilled on ice. Another 0.5 unit of reverse transcriptase was added and RT reaction repeated once more. To amplify the cDNAs, 1 µl of above RT product was mixed with the degenerate primers (100-200 ng each), dNTPs, 10xPCR buffer, H<sub>2</sub>O and one unit of Taq polymerase to a final volume of 100 µl. Polymerase Chain Reaction (PCR) was carried out for 32 cycles (95°C, 30sec; 48°C, 1min; 72°C, 1.5min). A final extension step was performed at 72°C for 15 minutes. All materials were purchased from Boehringer Mannheim unless otherwise noted. Figure 2 below shows a result of a RT-PCR.

Figure 2, lane 1, 1Kb DNA ladder; lane 2, 435 cell RNA; lane 3, 231 cell RNA and lane 4, MCF-7 cell RNA. Each sample lane contains one fifth of the PCR reaction volume. The expected size of the PCR product is ~ 180bp which is marked with the arrow. This is a representative of three RT-PCR experiment repeats.





Sequence analysis revealed that the MCF-7 cell RNA contains one novel ADAM protein. I designated this novel ADAM as ADAMx. In addition, human homologue meltrin- $\alpha$  and metargidin was also found in MCF-7 cells. Several ADAMx gene-specific primers were synthesized according to the sequence information derived from these experiments. RT-PCR using the gene-specific primers for ADAMx confirmed that there was no detectable ADAMx messenger in the invasive 231 and 435 cells (not shown). ADAMx is also identified in a human osteosarcoma cell line (MG63, not shown).

## B.2. Cloning Full Length ADAMx Gene

I used two approaches to clone the full length ADAMx gene. One was the Rapid Amplification of cDNA ends (RACE) [23,24] and the second is to screen the human cDNA libraries. This was done by both me on an human placental cDNA library (Invitrogen) using the filter-lifting method (see below) and by a commercial service (Research Genetics, Huntsville, AL) on the human BCA library using high density membrane hybridization method.

### *B.2a. RACE experiments*

RACE (Rapid Amplification of cDNA Ends) was used in order to clone the rest of the sequence of ADAMx gene. To perform 3'-RACE, first, reverse transcription reactions were carried out using oligo dT<sub>20</sub> or oligo dT<sub>17</sub>-Ro where Ro is an adapter primer. Then, cDNA was amplified using an upstream ADAMx gene-specific primer and dT<sub>20</sub>VN or Ro. Often, nested PCR was necessary to further amplify gene specific products. In this case, another pair of primers inside the first pair were used and PCR was carried out as usual. To perform 5'-RACE, the reverse transcription reactions were carried out using a downstream ADAMx gene-specific primer. The cDNA was purified from the reaction mixture (to remove the first primer, RNA and enzymes). First the cDNA was treated with NaOH to denature all the enzymes and then neutralized by adding HCl. The cDNA was then purified by using the QIAquick Spin PCR Purification Kit (QIAGEN) according to manufacture's instruction or using the Microcon-30 concentrators (Microcon). The cDNA was then tailed with poly(A) nucleotide using dATP and terminal transferase (Promega) at 37°C for half an hour. The transferase was denatured by heating the reaction mixture at 65°C for 2 minutes. cDNA was then purified by Phenol/Chloroform extraction and ethanol precipitation. This pellet was brought up in TE buffer and was used in PCR experiments. Finally, double stranded DNA was synthesized using oligo dT<sub>17</sub>-Ro and a downstream gene-specific primer (gs-1). Subsequent nested PCR was performed using Ro and another gene-specific primer inside the first gs-1.

As a result of the RACE experiments, at least 80% of the ADAMx gene was cloned (this is based on the sequence comparison to other known ADAM genes). An alignment of the putative integrin-binding loop of several ADAM's disintegrin domains is shown in figure 3. Note that in ADAMx, RGD sequence that appears in metargidin (ADAM-15) is replaced by a SRS sequence.



Figure 3. Sequence alignment of "integrin binding loop" among several ADAMs. Here MG63/MCF7 represents ADAMx gene.

Metargidin  
MG63/MCF7  
Human Meltrin- $\alpha$   
Mouse Meltrin- $\alpha$

\*\*\*  
-CRPTRGDC-  
-CCPSSRSC-  
-CRDSSNSC-  
-CRDSSNSC-

The confirmed protein sequence of ADAMx is shown in figure 4 (below).

....LCQHPALWKNQVALEEAKIKFQTWAPQKWNRLRLGLVPGPSCIRLEILMLLVIFVPSMYC  
HLGSIYYSFYEIIPKRLTVQGGDSPVEGLSYLLLMQGQKHLVHLKVKRNHVNNFPVYSY  
HNGLLGQESPFISHDCHYEGYIEGMSGSFVSVNICAGLRGTSSLRRKNLTALSPWTLQD  
GLNMCYTPTWHIKRESPVSTSWQQGSRKPHDLQALSYLCSHKKYVEMFVVNNQRFQ  
MWGSNVNETVQTVVDIALANSFTRGINTEVVLAGEIWTEDLIDVTVDLQITLRNFNHW  
RQEMFFHRAKHDVAHMIVGHHPGQNMGQAFLSGACSSGFAAAVESFHHEDVLLFAALMA  
HELGHNLGIQHDHSACFCKDKHFCMLHENITKESGFSSCSSDYFYQFLREHKGACLFN  
**KPRPRSRKRRDSACGNGVVEDTEQCDGSLCQHHACCDENCILKAKAECSDGPCCHKCK**  
**FHRKGYPCCPSSRSCDLPEFCNGTSALCPNNRHKQDGSKCHTIYECLKVHCMDPNNQCL**  
QLYGYGAKSASQECYNMNSKGDQFGNCGISTSPGSQYVRCSDGNIFCGKLICSGITGL  
PKINLQHTMIQVPQGDGSCWSMDAYMSTDIPDEGDVHNGTYCAPNKVCLNSACTDKTP  
VISACNPKKTCNGKGV CNDLGHCHCNEGHAPPDCVTAGSGGSVD SGLPGKLG GTPSG  
EGENHNMTHSRREEHAVDMMILSFILFILLSTII\*SACLKNHQRLPRQKFLQQWLHHR  
PQK \*SQKQQKWPQKKKKKHMPWT

Figure 4. The sequence of ADAMx (incomplete). The zinc-binding motif in the metalloprotease domain is double underlined. The disintegrin domain is in bold. The potential fusion peptide is underlined and the putative transmembrane region is in italics. The 3' coding region, 5' coding region and untranslated region of the gene remain to be completed.

The functional motifs/domains of ADAMx were identified by comparison of the sequence to those of known ADAMs. Sequence homology search using the NCBI BLAST Search showed that ADAMx has the highest protein sequence homology with

the fertilin molecules (ADAM-1) from mouse, guinea pig and macaque [25]. In some regions, ADAMx's amino acid sequence is 50-70% identical to the fertilin molecules. An alignment of partial sequence of ADAMx with fertilin alpha molecules is shown (see attachment). Therefore, ADAMx seems to be a human homologue of fertilin alpha.

The above sequence information revealed that ADAMx contains a pro-domain, a metalloprotease domain represented by the presence of the conserved Zn-binding peptide sequence HELGHNLGIQH; The MP domain is followed by a disintegrin domain, a cysteine-rich domain, an EGF-like domain and a transmembrane domain (Figure 5). The TM region is predicted by hydropathy plot analysis (Figure 6).

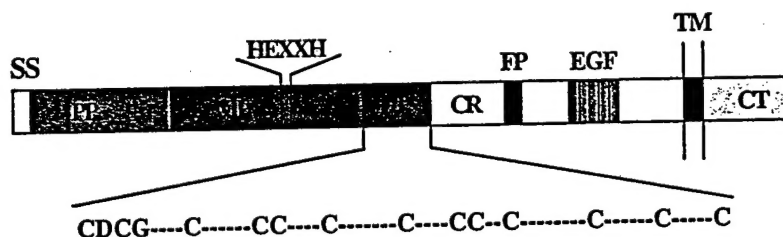


Figure 5. Domain arrangement of ADAMx

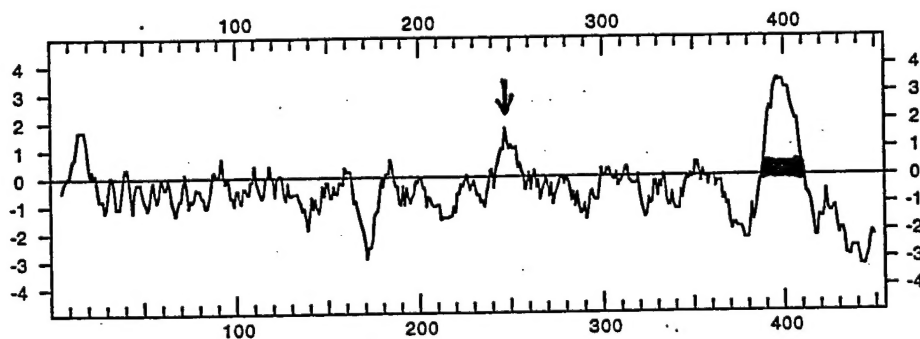


Figure 6. Kyte Doolittle hydropathy plot of ADAMx (partial sequence). The potential fusion peptide is marked by an arrow and the predicted transmembrane region is blackened.

### B.2b. Library screening

Double stranded DNAs of ADAMx encoding either the disintegrin domain of ADAMx or the disintegrin domain plus 5' end of the cysteine-rich domain were amplified by PCR and gel-purified twice. These samples were used to synthesize the [ $\alpha$ ]- $^{32}$ P-radio-labeled DNA probes using the Random Primed DNA Labeling Kit (Boehringer Mannheim) according to manufacturer's recommendation. The human placental cDNA library in *E.coli* (MC1061/P3) was titered and the appropriate amount of the bacteria culture was plated on LB-Amp agarose. Overnight colonies were transferred to membrane filters (Millipore, 0.45 $\mu$ M) by filter-lifting. The colonies on the filters were washed, lysed, fixed and then cross-linked to the membrane by an UV Crosslinker. The filters were allowed to hybridize with either of the two labeled DNA probe at 68°C overnight in the "QuikHyb" solution (Stratagene). The filters were then washed by buffers of low stringency and then high stringency. The dried filters were exposed to the X-ray film (Kodak) for autoradiography. At least 18 positive clones were picked and the corresponding bacterial culture grown from which second round library screening were conducted. After this, however, no positive clones were found.

Meanwhile, the same [ $\alpha$ ]- $^{32}$ P-radio-labeled DNA probes described above were sent to a commercial company (Research Genetics, Huntsville, AL). The human BCA cDNA libraries were screened by high-density membrane hybridization. After several attempts, they were able to identify four I.M.A.G.E. clones that were positively hybridized to my DNA probes. However, PCR amplification of these clones using ADAMx-specific primers yielded negative results. Therefore, these clones must be non-specific hybrids. The above results strongly suggest that ADAMx is a rare gene. Next, I plan to screen a genomic DNA library and/or screening MCF-7 cDNA library in order to obtain complete sequence of ADAMx.

### B.3. Polyclonal antisera production

I have raised polyclonal antibodies against the disintegrin domain of ADAMx. To make the antigen, the DNA of DIS domain was PCR amplified with primers each containing a restriction enzyme cleavage site. The PCR product was cloned into expression vector pET15b (Novagen) using restriction sites Xho I and BamH I. The protein was expressed in *E. coli* bacterial BL21 (DE3) cells upon IPTG induction. The recombinant protein was found to be retained in the inclusion bodies and thus had to be solubilized by using 6M urea. The protein contains a N-terminal His-tag and was coupled to a Ni $^{2+}$ -affinity Sepharose 6B column (Figure 7). The protein was eluted off using 1M imidazole in 6 M urea/PBS. The sample was dialyzed against 6 M urea/PBS to remove imidazole.



Figure 7. 15% reducing SDS-PAGE analysis of His-tagged ADAMx protein by  $\text{Ni}^{2+}$  resin chromatography. Lane 1, protein markers; Lane 2 and 3, ADAMx eluted from the affinity column.

The purified and concentrated antigen was used to immunize rabbits. Briefly, 150 $\mu\text{g}$  antigen was mixed with Complete Freund's Adjuvant and was injected into each New Zealand white rabbit. Pre-bleed sera were collected prior to the injection. The rabbits were immunized six more times with 100 $\mu\text{g}$  of antigen every two weeks.

The ability of the antisera to recognize recombinant disintegrin domain of ADAMx was confirmed by ELISA. The disintegrin domain of ADAM-15 [26] was used to examine cross reactivity (Figure 8).

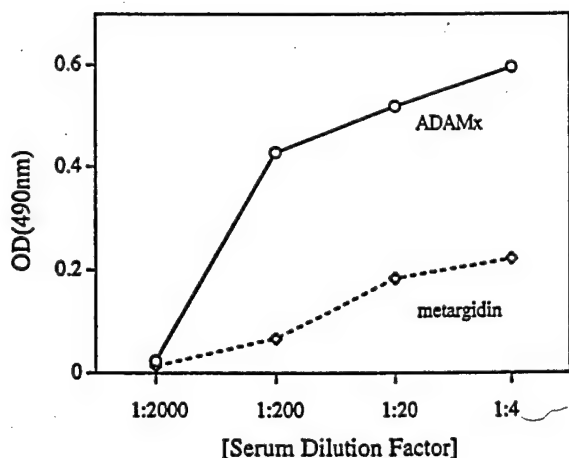


Figure 8. ELISA measurement of the specificity and activity of polyclonal antibodies raised against the disintegrin domain of ADAMx. The disintegrin domain of ADAM-15, metargidin, was used as a control. Recognition of the antigens was accessed by optical density at 490nm for the reaction of o-phenylenediamine.

### C. Discussion

This project focuses on the study of a novel gene, ADAMx, found in human breast tumor MCF-7 cells. ADAMx's partial protein sequence reveals that it constitutes the following domains characteristic of the ADAM proteins: a prodomain, a metalloprotease domain that contains the zinc-binding motif, a disintegrin domain comprising highly conserved cysteine arrangement pattern found in all soluble snake venom disintegrins and previously identified ADAMs, a cysteine-rich domain, a fusion

peptide, a well defined EGF domain and a putative transmembrane domain. So far, experiments have focused on DNA cloning and antibody production.

It is known that soluble metalloproteases (MMPs) and membrane-type metalloproteases (MT-MMPs) are involved in basement membrane degradation, cell growth regulation and tumor invasion. The snake venom peptide disintegrins are ligands of cell surface receptor integrins, a family of proteins intimately involved in ECM turnover and tumorigenesis. Based on the fact that ADAMx has a disintegrin domain, my hypothesis is that, by interfering the interaction between cell surface integrin and ECM proteins, the ADAMx's disintegrin domain may alter adhesion and migration properties of tumor cells.

Interestingly, ADAMx is found in the non-invasive MCF-7 cells that retain epithelial morphology whereas it is not expressed in MDA-MB-231 and MDA-MB-435 breast carcinoma cells, which have lost epithelial phenotype and are highly invasive. This expression pattern is opposite to that of MMPs and MT-MMPs, both of which are highly expressed in the two invasive breast carcinoma cell lines. This differential expression pattern suggests that ADAMx may be important for maintaining epithelial morphology. This is indicative that ADAMx may be important in controlling cell growth, proliferation.

Together, ADAMx may be a key determinant in the invasive and metastatic potential of breast epithelial carcinoma.

#### **D. Future Plans**

I will devote my effort in the following studies to generate information about ADAMx.

- 1) complete the cloning and sequencing of ADAMx. This includes the confirmation of the 3' and 5' coding regions of the protein.
- 2) express individual domains of ADAMx, especially the active disintegrin domain in the baculovirus/insect cell system. This will provide powerful reagent for the study its binding to integrins.
- 3) assess cell type and tissue specificity of ADAMx gene and gene product by Northern and Western blotting.
- 4) study the role of ADAMx in cell adhesion and migration by using the ADAMx-transfected MDA-MB-435 cells.

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# MAP Multiple Sequence Alignment Results

Page 1.1

	1	15	16	30	31	45	46	60	61	75	76	90
1 mouse	---	---	---	---	---	---	---	---	---	---	---	---
2 GP	MRS	GSM	MA	SV	RNT	IS	FS	AS	LQ	KA	HV	LV
3 Adam-X	---	---	---	---	---	---	---	---	---	---	---	---
4 Monk	---	---	---	---	---	---	---	---	---	---	---	---
5 rabbit	---	---	---	---	---	---	---	---	---	---	---	---

Page 2.1

	91	105	106	120	121	135	136	150	151	165	166	180
1 mouse	---	---	---	---	---	---	---	---	---	---	---	---
2 GP	LT	V	KG	SQ	DP	GG	RT	SY	ML	L	I	Q
3 Adam-X	---	---	---	---	---	---	---	---	---	---	---	---
4 Monk	---	---	---	---	---	---	---	---	---	---	---	---
5 rabbit	---	---	---	---	---	---	---	---	---	---	---	---

Page 3.1

	181	195	196	210	211	225	226	240	241	255	256	270
1 mouse	---	---	---	---	---	---	---	---	---	---	---	---
2 GP	EN	TS	Y	G	IE	P	IL	SS	QR	FE	H	V
3 Adam-X	---	---	---	---	---	---	---	---	---	---	---	---
4 Monk	---	---	---	---	---	---	---	---	---	---	---	---
5 rabbit	---	---	---	---	---	---	---	---	---	---	---	---

Page 4.1

	271	285	286	300	301	315	316	330	331	345	346	360
1 mouse	---	---	---	---	---	---	---	---	---	---	---	---
2 GP	VQ	AV	MD	I	I	A	L	A	N	S	F	T
3 Adam-X	---	---	---	---	---	---	---	---	---	---	---	---
4 Monk	---	---	---	---	---	---	---	---	---	---	---	---

5 rabbit VQVRMDIIALANSET RGINTEVWLAGMEIW TEGDLTEVAADLQVT LRNFNSWRQEQLVHR VRHDVAHMIVGRHPG ENTGOAFLNGACSSG

Page 5.1

1 mouse 361 375 376 390 391 405 406 420 421 435 436 450  
 2 GP FAAAVEAFHHEDVLL FAALMAHELGHNLGI QHDHPTCTCGPKHFC LMGEKIGKDSGFSNC SSDHFLRFLHDHGA CLLDEPGRQSRMRA  
 3 Adam-X FAAAVEAFHHEDALL SAALLVHELGHNLGI RHDHSACVCRDKHSC LMQENITEESGFSNC SSDYFYHFLHEHGA CLFNKPWHKARRRA  
 4 Monk FAAAVESFHEDVLL FAALMAHELGHNLGI QHDHSACFCCKDKHFC LMHENITKESGFSNC SSDYFYQFLREHGA CLFNKPRPRSRKRRD  
 5 rabbit FAAAVESFHEDMLL FAALMVHELGHNLGI QHDHSACFCREKHFC LMHENITKESGFSNC SSDYFHQFLREHGA CLFNKPRPRGRKRRD  
 6 rabbit FAAAVESFHEDILL FAALMAHELGHNLGI QHDHSACTCKNQPF C LMGENITKESFSNC SSDDFYRFLREHGA CLFNKPRHRSRTRRL

Page 6.1

1 mouse 451 465 466 480 481 495 496 510 511 525 526 540  
 2 GP ANCGNGVEDLEED CGSDCDHPCCSPTC TLKEGAQCSEGLCCY NCTFKKGSICRPAC DVCDLPEYCDGSTQE CPANSIMQDGTQCDR  
 3 Adam-X ATCGNGVVEEQCD CGVNCDTSECCDQAC NLKGNATCSNELCCS DCQYKNSGYLCRPSV GPCDLPEYCTGQSGK CPLDTYKQDGTGTCNE  
 4 Monk SACNGNGVEDTEQCD CGSLCQHHACDENC ILKAKAECSDBGPCCH KCKFHRKGYPCCPSS RSCDLPEFCNGTSAL CPNNRHKQDGSKCHT  
 5 rabbit SRCNGNGVETPEQCD CGSDCALDPCCDSMC RLKDNAQCGYGLCCF RCKYRRKGFLCRSIR GNCDLPEYCSGKSAS CPPDAYKQDGTGTCDR

Page 7.1

1 mouse 541 555 556 570 571 585 586 600 601 615 616 630  
 2 GP IYYCLGGWCKNPDQ CSRIYGYPARSAPEE CYISVNTKANRFGNC GHITSANFRYETCS DEDVFCGLVCTDVRY LPKVKPLHSLLOVPY  
 3 Adam-X GFFCVSKGCTDPGIQ CATYFGHGARSAPDA CYTTLNSIGNIFGNC GQSGNP-TTYVGCSG DSTKCGKLICTGISS IPPIRALFAAIIQIPH  
 4 Monk IYECCLKVHCMDPNNQ CLQLYGYGAKSASOE CYNSMNSKGDQFGNC GISTSPGSQYVRCSD GNIFCGKLICTGITG LPKINLQHTMIQVPO  
 5 rabbit VYRCLGGQCMNPDQ CSNIYGIPARSAPEE CYVLMNSKGRDFGNC GSPPALQSSYVPCAD ENIFCGKLICTEVKL LPQILPQHTVIOVAY

Page 8.1

1 mouse 631 645 646 660 661 675 676 690 691 705 706 720  
 2 GP GEDWCWSMDAYNI-T DVPDDGDVQSGSFA PNKVCMEYICTGRGV LOYNCEPQEMCHNG VCNFKKHCHCDAGFA PDCSSPGNGGSVDS  
 3 Adam-X GDDWCWSISNFGDPA SSFTEGAVSAGTS CA SGKACVNAQCSTFTL DTANCSAAEMCNENG ICNNLGHCHCGDGA PPNCKEQGTGGSIDS  
 4 Monk GDGSCWSMDAYMS-T DIPDEGDVHNGTYCA PNKVCILNSACTDKTP VISACNPKKTCNGKG VCNDLGHCHCNEGHA PPDC-TAGSGGSVDS  
 5 rabbit EDDWCWSIDSN---S GCSIDYGDVQRNTYCA LNKVCKDHSVYQA PNSDCQADEMCSGKG VCNFRHCHCDSGYA PPDCRNPGTGGSVDS

## Page 9.1

	721	735	736	750	751	765	766	780	781	795	796	810
1 mouse	GPVGKPADRHLSLF	LAEEPPDDKMEDEEV	NLKVMVLVPIFLVV	LLCCIMLIAYLWSEV	QEVVSPSSSESSSS	SSWSDSDSQ						
2 GP	GPPPP--SS-TPTA	PKPKQTTKASSENIL	ALIGLIILVILLILL	VICAICL								
3 Adam-X	GLPGKLGTF-SGEG	ENHNMTSRREEHAV	DMILSFII-LFIIL	LSTIIS								
4 Monk	GPPGMQVTNN-SESG	SE-SIARGQSLRQDV	DYKLVLVLPFLVL	LLCSLLTISYLCSEV	QTAVAEVEESSTETT	LESE-LTSADLV						
5 rabbit	GPPGKPYNRN-ISSS	TN-S-SRITKKKKSE	NLNVLFVFWPIFLII	VLCVLIILSYLWSEV	KSVVVSIAESKEKSE	ESSEELPSEESVEAP						

## Page 10.1

	811	825	826	840	841	855	856	870	871	885	886	900
1 mouse												
2 GP												
3 Adam-X					ACLKNHQRLPRQKFL	QQWLHHRPQKSQKQQ	KWPQKQKKKHMPT-					-GI
4 Monk					AQPGGEETLPPGE--	-----EAQPGGEETL	PPGEAPPAEAPPA	EA				-PPPEAA
			PIAE	EILPPGEEAPPGEE								
5 rabbit	PPEOPAQOOEAPQQQ	EA-PPAREAPP--E	AARPAEAP-PPPE--					QAPPP--EQA	PPPEAPKPAEAPPPP	EAAPPPQAPPPPEAA		

## Page 11.1

	901	915	916	930	931	945	946	960	961	975	976	990
1 mouse	-	-	-	-	-	-	-	-	-	-	-	-
2 GP	PAEEA	PPPEEEEAG	ELEEEPEPEPEEEE	EA	AFFED-	-	-	-	-	-	-	-
3 Adam-X	-	-	-	-	-	-	-	-	-	-	-	-
4 Monk	PPPEA	APPPEAAAP	EAAPA	EEAAA	PQAP	PP-EA	APPEA	PPAQ	-	-	-	-
5 rabbit	pppea	pppppeaaapp	eappppeaa	apppeap	pppea	pppppea	ppppp	q	-	-	-	-

### Alignment Data (Fasta format)

```
>mouse
-----AMEHQVVVCSVTPKDS PG-DTSHPPRSRKPDDLLLVLT
W-WSHTKYVEMFVVVNHQRFQWVGSNINETVQAVMDIIALANSFTRGINTEVVLVGLGLEIW
TEGDP I E V P V D L Q T T L R N F N F W R Q E K L V G R V R H D V A H L I V G H R P G E N E G Q A F L R G A C S G E
F A A A V E A F H H E D V L L F A A L M A H E L G N L G I Q H D H P T C T C G P K H F C L M G E K I G K D S G F S N C
S S D H F L R F L H D R G A C L L D E P G R Q S R M R R A A N C G N G V E D L E E C D G S C D C S H P C C S P T C
T L K E G A Q C S E G L C Y N C T F K K G S L C R P A E D V C D L P E Y C D G S T Q E C P A N S I M Q D G T Q C D R
I Y Y C L G G W C K N P D K Q C S R I Y G Y P A R S A P E E C Y I S V N T K A N R F G N C G H P T S A N F R Y E T C S D
E D V F C G K L V C T D V R Y L P K V K P L H S L L Q V P Y G E D W C W S M D A Y N I - T D V P D D G D V Q S G S F C A
P N K V C M E Y I C T G R G V I O Y N C E P O E M C H G N G V C N N F K H C H C D A G F A P P D C S S P G N G G S V D S
```

GPVKGKADRRHLSLSELAEEESPDDKMEDEEVNLMVVLVVPFIPLVLLCCIMLIAYLWSEV  
QEVVSPPESSSSSSWSDSQ-----

>GP

MRSGSMASVRNTISFSASLQKAHVVLHVARSLLOTCTLLMVAURLGLVPGHLCVRLVTK  
LLVGTVLLPHIHCHLGPVHYSSYEIIVPELTVKGSQDPGGRTSYMLLIQGHKQLIHLKV  
KRDFVDDFPVSYHNGVNRQETPSIARDCHYEGYIEGASSFVSACSGLRGI--LIK  
ENTSYGIEPILSSQRFHEVLVYMARQAPVSCRASAKDSQAVSTSWQQGSRKPHSVQALSS  
YLWVHTKYVEMFVVVNNQRFQWGSVDNETVQRVVDIIALANIIFTRGINTEVVLAGEVW  
TEGDLEVPVLDRLVRNFRNRQDKLLPRVRHDVHMIIVGHPGETSGQAFNLGACSSG  
FAAAVEAFHEDALLSALLVHELGNLGIHRHDHSAVCVRDKHSCLMQENITEESGFNSC  
SSDYFYHFLHEHRGACLFNKPWHKARRRRAATCGNGVVEESEQCDGCVNCDTSECCDQAC  
NLGNATCSNELCCSDCOYKNSGYLCRPSVGPCDLPEYCTGQSGKCPDLYKQDGTPCNE  
GFFCVSKGCTDPIQCATYFGHGARSAPDACYTTLNSIGNIFGNCGQSGNP-TTYVGCSSG  
DSTKCGKLICTGISSIPPIRALFAAIQIPHGDDWCWISINFGDPASSPTEGAVSAGTSCA  
SGKACVNAQCSTFTLDTANCSSAAEMCENGINCNLGHCHCGDGFAPPNCKEQGTGGSIDS  
GPPPP---SS-TPTAPPKPTQTTKASSENALIGLIILVILLLLVICAICL-----

-----GI

PAEEAPPPPEEEAGELEEEPEPEPEPEEEEEEEED-----

>Adam-X

MLL-VIFVPSMYCHLGSIIYFYEIIIPKRLTVQGGDSPVEGLSYLLMQGKHVHLKV  
KRNFVNFPVSYHNGLLGQESPFISHDCHYEGYIEGMSGSFVSVNICAGLRGTSSLIK  
EEKSYIEPMDSSRRFEHLVYTMHQ-----

TEGLIDVTVDLQITLRFNHNWQEMFFHRAKHDVAHMIVGHHPGQNMGOAFLSGACSSG  
FAAAVESFHEDVLLFAALMAHELGNLGIQHDHSAFCCKDKHFCMLHENITKESGFSSC  
SSDYFYQFLREHKGACLFNKPRLSRKRDSACNGVVEDTEQCDGSLCQHHACCDENC  
ILKAKAECSDGPCCHKCKFRKGYPCPSSRSCDLPEFCNGTSALCPNNRHKKQDGSKCHT  
IYECLKVHMDPNQCLQLYGYGAKSASQECYNSMNSKGDQFGNCGISTSPGSQYVRCSD  
GNIFCGKLI CSGITGLPKINLQHTMIQVPGDGSWSMDAYMS-TDIPDEGDVHNGTYCA  
PNKVCNLNSACTDKTPVISACNPKKTGKGVNCLGHCHCNEGHAPPDC-TAGSGGSVDS  
GLPGKLGTP-SGEGENHNMTSHRREHVAVDMMLISFII-LFIILLSTIIS-----

ACLKNHQLRPRQKFLQQLHHRPKSQKQKQKWPQKKKKHMPWT-----

>Monk

---MSVLALLKDSANILLYLWKSQVALEEVKIKFQTWAPQKWNLRMLVPGPSCIRLEIL  
LLL-VIFVPSMCHLGSIIYFYEIIIPKRLTVQGGSSVEGLSYLLFMQGHVHLKV  
KRSHFVNFPVSYHNGILGQESPFISHDCHYEGYIEGVSQSF-SVNTCAGLRGI--LIK  
EEKSYIEPMDSSRRFEHLVYTMAREARVSCGVTSDSHVSTSWQQGSRKPHDLQALS-  
YLWSTKYVEMFVVVNNQRFQWGSNINETVRVVDVIALAN-FTRGINTEVVLAGEIWI  
TEGDLDVAVDLQITLRFNHNWQEMLFRRAKHDVAHMIVGHHPGQNTGQAFLSGACSSG  
FAAAVESFHEDMLLFAALMVHELGNLGIQHDHSAFCREKHFCLMHENITKESGFNSC  
SSDYFHQFLREHKGACLFNKPRLGRKRDSACNGVVEDTEECDCGSACHLDPCCDPTC

```

TLKEHAECSHGLCCLDCTFRKRGFLCRPTQDECDLPEYCDGSSAECPADSYKQDGTLCDR
IHYCSGGCKNDQCNVNIYGYPARSAPEDCYISNTRGDRFGNCGHPTDQQTYYVTCSD
DNVFCGKLICTGVQLPRVKAHTVIQVPHDNDWCWMDADNI-TDTPDNGNVHVGTSKA
PNKVCITYSCVHHSILLYDCRPEESCHGKGVNLRHCHCESGFAPDCKNPGNGGSGVDS
GPPGMQVTNN-SESGSE-SIARGQSLRQDVYKLVVLLVPLFLVLLCLSLTISYLCSEV
QTAVAEVEESSTETTLESE-LTSADLV-----PIAEIILPPGEEAPPPEEE
APQPGEETLPPGE-----EAPQGEETLPPGEAPPAAEAAPAAEA-----PPPEAA
PPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPPEAAPQ-
>rabbit
---MLATTSARVSSSSLYSFQPHMVLNGAARAPQSWVPQMNGRLGLVPGLSRVRLGTM
LLWGMTFLPSIYMEI--VHYSSYEMVIPESLITVEGSEKPGEKASYILFMQGQKQLVHLHV
KRDFVDDFPVSYHNRLVQEMLFISRNCCYEGYIDGVPGSFVSNTCSGLRGV--LVK
GETSYSEPIILSSKRFEHALYTMAGAHVSCSVTSKGGQGMSTSRQQGSRKLNHPQALS-
YLSHTKSVEMFVVDNQRFQWGRNVSETVQRVMDIIALANSFTRGINTEVVLAGMEIW
TEGDLTEVAADLQVTLRNFNSWRQEQVLRVVRHDVAHMIVGRHPGENTGQAFNLGACSSG
FAAAVESFHEDILLFAALMAHELGNLGIQHDHSACTCKNQPFCLMGENITKESFSNC
SSDDFYRFLREHRGACLFNKPRLRSRTRRLSRGNGVETPEQDCGSDCALDPCCDSDMC
RLKDNAQCGYGLCCFRCKYRKGFLLCSIRGNCDLPEYCSGKSASCPDAYKQDGTPCDR
VYRCLGGQCMNPKQCSNIYGIPARSAPEECYVLMNSKGRFGNGCGSPPALQSSYVPCAD
ENIFCGKLICTEVKLLPQILPQHTVIQVAYEDDWCSIDSN---SGCSDYGDVQRNTYCA
LNKVKCDHSCVVYQAPNSDCQADEMCSGKGVNFRHCHCDSGYAPDCRNPGTGGSVDS
GPPGKPYNRN-ISSSTN-S-SRITKKKSENLVFFVVFIFLIIVLCVLIILSYLWSEV
KSVVVSIAESKEESEESEELPSEESVEAPPPEQPAQQQEAAPQQA-PPAREAPPP--E
AARPAEAP-PPPE-----QAPPP--EQAPPPPEAPKPAEAPPPEAAPPPQAPPPPEAA
PPPEAAPPPPEAAPPPPEAAPPPPEAAPPPPEAAPPPPEAAPPPPEAAPPPPE

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